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Original investigation

Recent recovery of the Italian wolf population: a genetic investigation using microsatellites

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Abstract

Genetic differentiation within the Italian wolf population was investigated by microsatellite analysis of 38 individuals from 4 distinct sampling sites of the current wolf range throughout the peninsula. A set of 6 microsatellite loci was used, which showed a high level of polymorphism and a combined probability of identity ranging from 10^{-4} to 10^{-6}. The overall DNA variability detected was considerable and only slightly lower than that found for North American grey wolves.
The two largest Italian subpopulations taken into consideration, Tuscan Apennines and central-southern Apennines, proved moderately divergent, and data are consistent with a derivation of the western Alps subpopulation from the former, while the latter showed close similarity to the western coast subpopulation. Gene flow was relatively high across the Italian population and the presence of isolation by distance was supported by our data, as measures of genetic distance were consistent with geographical distribution of sampling sites. High levels of divergence were found between Italian and other European samples. These findings suggest that, despite their absolute mtDNA monomorphism, Italian wolves have preserved a high nuclear DNA heterogeneity and a well-defined genetic identity. A further enlargement of range, which can be expected on the basis of extensive wolf dispersal, might cancel their historical isolation in a few decades, thus favouring a genetic exchange with the east European gene pool.

Key words: *Canis lupus*, microsatellites, variability, population structure, Italy

Introduction

Since the end of the 19th century large predator populations have declined in Italy due to progressive habitat disruption and to direct persecution by humans. As a consequence, different species approached extinction (i. e., brown bear, wolf, and lynx). Changes in human activities, wildlife and wood management, and public opinion have led to the restoration of more favourable environmental conditions and to increased protection of these species. Due to such improvements, in the last 30 years an important predator species, the wolf (*Canis lupus*), has increased in number and enlarged its range (Francisci and Guberti 1993; Boitani and Ciucci 1993; Meriggi

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and Lovari 1996). Wolves in the Italian environment play a key role in wild communities, being the only well-distributed large mammals preying mostly on wild ungulates (Mattioli et al. 1995). During the last two centuries the Italian population became isolated from other European populations, due to the extirpation of the species throughout the Alps (Cagnolaro et al. 1974). The northern border of its range initially moved southwards, towards the central regions, and the presence was restricted to the less accessible areas of the Apennines and to a wooded area along the Tyrrhenian coast (Cagnolaro et al. 1974; Zimen and Boitani 1975). In 1973, the number of wolves inhabiting the Italian peninsula was estimated to be approximately 100 individuals (Zimen and Boitani 1975), after which the population recovered, reaching an estimated size of 400–500 individuals (Boitani 1992).

The demographic recovery of the species was accompanied by a northward expansion of its range, which during the last ten years led to the recolonization of the western Alps, and to the consequent appearance of the wolf in France (Breitenmoser 1998).

The effects of population decline and the subsequent range expansion in the genetic diversity of the Italian wolf population were studied by allozyme and mitochondrial DNA (mtDNA) analyses (Randi et al. 1993; Wayne et al. 1992; Randi et al. 1995; Vilà et al. 1999; Randi et al. 2000). Randi and co-workers (1993), using a set of 40 allozymes over a sample of 38 wolves, found a level of polymorphism and heterozygosity comparable to that of larger North American populations (Kennedy et al. 1991). On the other hand, mtDNA consensus sequences revealed the presence of a single haplotype in all the sampled Italian wolves. This apparent contradiction probably results from the different inheritance systems, based exclusively on female mtDNA transmission.

In order to investigate the actual effects of the population bottleneck and fragmentation on the genetic structure and variability of wolves, nuclear genetic markers, e. g. microsatellites, are more informative. Different studies carried out on North American wolf populations demonstrated the effectiveness of microsatellite loci as a molecular tool for assessing population structure parameters (Roy et al. 1994; Forbes and Boyd 1997), genotyping animals for reintroduction programmes (Garcia-Moreno et al. 1996), evaluating relatedness among individuals (Smith et al. 1996), and estimating genetic variation following natural colonizations (Forbes and Boyd 1996).

The aims of the present study were: 1) to reconstruct the dynamics of the Italian wolf recovery by comparing the genetic pattern of different subpopulations; 2) to evaluate nuclear DNA diversity among and within sub-populations; 3) to estimate the degree of gene flow among different areas. Wolves from historical "stronghold areas" and from recent colonized regions were sampled for comparison.

Material and methods

Sample description and collection

Italian wolf samples were collected from four regions (Fig. 1):

AR – Tuscan Apennines, which probably represented the northern border of the Italian wolf range along the Apennines for half a century (Cagnolaro et al. 1974);

AB – Central-southern Apennines, the part of Italy where the species has always been present in historic times;

VC – Alta Maremma, where the presence of the species was always recorded during the last century, but the area was characterized by continuous new settlements of breeding packs followed by complete or partial eradication (illegal killing);

FR – Alpes Maritimes, France, originating in the early 1990s from individuals moving across from Italy (Randi et al. 2000). Its present size is approximately 20–30 units, of which dispersing individuals are colonizing new areas of the western Alps.

Seventeen tissue samples came from illegally killed wolves, recovered before 1992 in central and southern Italy and obtained from Prof. G. B. Hartl (University of Kiel, Germany). Thirteen specimens are from the northern Apennines, mostly the province of Arezzo: 12 (3 tissues and 9 hairs), supplied by the Provincial Administra-

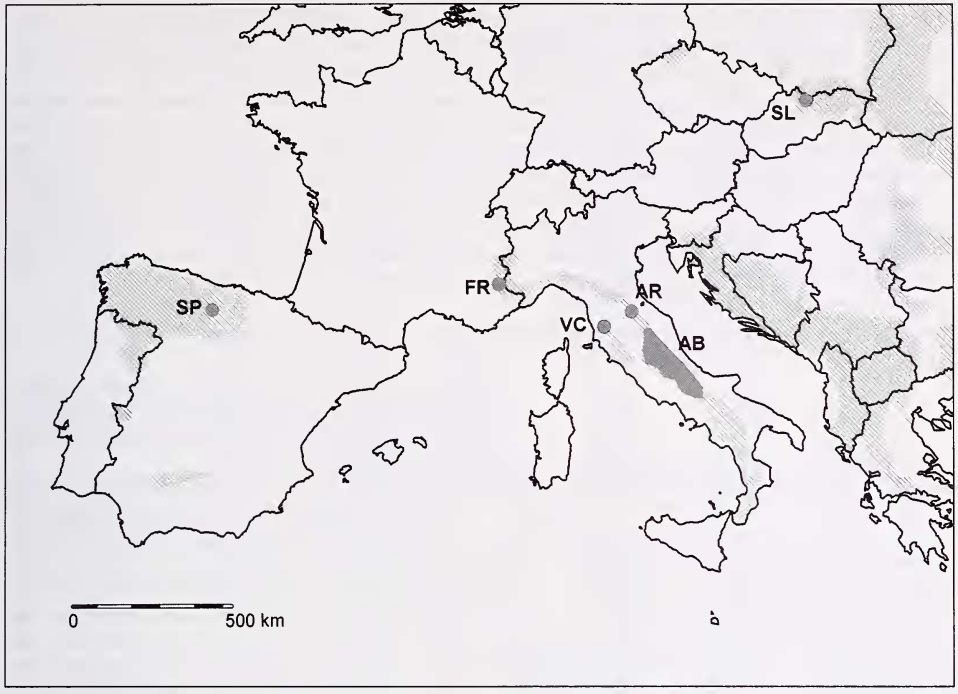

Fig. 1. Geographical location of sampling sites (solid grey) and present distribution range of wolves (hatching).

tion of Arezzo (Tuscany) or by the Corpo Forestale dello Stato, derived from animals dying in the period 1991–1999, whereas one hair sample was collected in the field during a survey in the Foreste Casentinesi National Park. Three samples of the Alta Maremma population were obtained from the Veterinary Service of Volterra (Pisa) and one hair sample was collected in a natural preserve near Volterra. For the Alpine population, 4 tissue samples were provided by Valiere Nathaniel (University of Grenoble, France). In addition, ten wolf samples from Asturias, Spain (SP) and three from Slovakia (SL) were analysed for comparison.

DNA isolation and amplification

Proteinase K digestion and phenol/chloroform standard protocols were used for genomic DNA isolation from tissues. Nuclear DNA was extracted from hair bulbs either according to Higuchi et al. (1988) or by Chelex isolation (Walsh et al. 1991). Samples were genotyped for five dinucleotide $(AC)_n$ polymorphic microsatellite loci and one tetranucleotide locus, previously characterized in dog (Ostrander et al. 1993; Francisco et al. 1996). Amplifications were carried out in 20 µl volume, containing 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 200 µM each dNTP, 0.5 units Taq DNA Polymerase (Promega), 5 pmol of each primer and 1–4 µl of DNA solution. An initial denaturation step at 94 °C for 3 min was followed by 35 cycles of amplification each of 45 sec at 92 °C, 45 sec at the annealing temperature (55–58 °C) and 60 sec at 72 °C. The reaction terminated with a polymerization step at 72 °C for 5 min.

In order to verify the successful products of single PCRs, 5 µl of reaction solution were run on a 2% agarose gel (Biorad) containing ethidium bromide, and the presence of correctly amplified fragments was detected by comparing their length with a DNA size marker.

Single alleles were sized by running denatured PCR products through capillary electrophoresis in an ABI PRISM 310 automatic sequencer (Perkin-Elmer).

Statistical analysis

By combining alleles at each locus, individual genotypes were obtained. The GENEPOP soft-

ware program version 3.2 a (Raymond and Rousset 1995) was used to calculate allele frequencies and to test data sets for deviation from Hardy-Weinberg equilibrium (HWE) as well as for genotypic linkage disequilibrium. Because many rare alleles were present, HWE departures were also tested "with pooling" (Hartl and Clark 1989), by grouping genotypes into three classes: homozygotes for the most common allele, common/rare allele heterozygotes, and all other genotypes.

Observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) (Nei 1978) were estimated for each subpopulation. Probability of genotype identity was obtained using the formula

$$\sum_i p_i^4 + \sum_i \sum_{j>i} (2p_i p_j)^2$$

where p_i and p_j are the frequencies of the ith and jth alleles at a given locus (Paetkau and Strobeck 1994). Single locus probabilities were combined to obtain the total probability over all 6 loci, assuming independence of different loci, as supported by the microsatellite linkage map in the domestic dog (Mellersh et al. 1997).

In order to evaluate the level of genetic variation, the H_e estimated for the overall Italian population ($N = 38$) over five of the six examined loci (109, 123, 204, 250, and 377) was compared with values recalculated for North American populations over the same loci using published data (Roy et al. 1994; Forbes and Boyd 1997).

Allelic and genotypic differentiations were evaluated for each population pair within the Italian range (AR, AB, VC, and FR), and then were pooled and compared with the two other European populations (SP and SL). Two different approaches were used to estimate the level of differentiation between samples by GENEPOP: a Fisher exact test was performed to test the homogeneity of allelic distributions across populations (Raymond and Rousset 1995), whereas a log-likelihood (G) based exact test was used for genotypic differentiation (Goudet et al. 1996). The significance level was always established using Bonferroni's criterion for multiple tests. In both cases, an unbiased estimate of the p-value was obtained, associated with the null hypothesis of identical distribution across populations.

A matrix was created containing the proportions of shared alleles (P_{AS}), over the six loci, for all pairwise comparisons of sampled individuals, as described in Bowcock et al. (1994). In order to obtain a measure of divergence among populations, P_{AS} values were averaged over population pairs and the pairwise distance value D_{AS} was calculated as

$$(1 - \overline{P}_{AS\,ij})$$

where the second term represents the mean P_{AS} calculated over all combinations between the ith and the jth subpopulation genotypes. Mean distance values were also computed among individuals of a single sample, in order to evaluate intra-group homogeneity. To eliminate the bias originating from different degrees of sample homogeneity, mostly due to different breadths of sampling areas, a new matrix was extrapolated, averaging the differences between inter- and intra-population distance values:

$$D'_{AS\,ij} = \frac{(D_{AS\,ij} - D_{AS\,ii}) + (D_{AS\,ij} - D_{AS\,jj})}{2}$$

Furthermore, Nei's unbiased genetic distance (Nei 1972) was computed by BIOSYS-2 software (Swofford and Selander 1997) between all subpopulation pairs.

Multilocus F-statistic was calculated by GENEPOP, estimating the F_{ST} coefficient for each pair of samples and for all Italian samples, according to Weir and Cockerham (1984). In order to evaluate gene flow among subpoputations, the same program allowed the effective number of migrants per generation (Nm) to be estimated on the basis of the private allele model (Slatkin 1985; Barton and Slatkin 1986). Thereafter a statistical analysis was carried out using the ISOLDE program, in the GENEPOP package, performing Mantel's tests (1 000 permutations) to highlight the possible presence of isolation by distance in the Italian population. For this purpose, D'_{AS}, Nei's unbiased distance, and F_{ST} were chosen as measures of genetic divergence and compared with geographic distance.

Results

A total of 51 wolves was genotyped at six microsatellite loci. All the loci showed polymorphism in the four Italian subpopulations investigated, except for locus 377 in the Alpine sample where allele A was fixed. Allele frequencies are given in table 1. Average H_e ranged from 0.505 ± 0.106 to 0.680 ± 0.038, while the probability of identity varied from 1/1 700 for the Alpine sample to less than 1/100 000 for the central-southern Apennines subpopulation (Tab. 2). In three out of four Italian samples, average H_e had a lower value than the observed one (Tab. 3), possibly due to limited outbreeding. Negative F_{is} values confirm this possibility (mean F_{is} over 6

Table 1. Allele frequency distributions at 6 microsatellite loci in wolf samples (AR – Tuscan Apennines; AB – Central-southern Apennines; VC – Alta Maremma: FR – Alpes Maritimes: SP – Spain; SL – Slovakia).

	AR	AB	VC	FR	SP	SL
Locus 109						
A	0.115	0.000	0.000	0.000	0.100	0.000
B	0.538	0.618	0.750	0.500	0.350	0.500
C	0.038	0.059	0.125	0.000	0.000	0.000
D	0.038	0.176	0.000	0.375	0.400	0.000
E	0.269	0.147	0.125	0.125	0.000	0.000
F	0.000	0.000	0.000	0.000	0.000	0.500
G	0.000	0.000	0.000	0.000	0.150	0.000
Locus 123						
A	0.240	0.353	0.000	0.000	0.000	0.000
B	0.000	0.059	0.125	0.125	0.000	0.000
C	0.160	0.206	0.375	0.000	0.278	0.667
D	0.080	0.000	0.000	0.250	0.056	0.000
E	0.000	0.029	0.000	0.250	0.389	0.333
F	0.520	0.324	0.500	0.375	0.167	0.000
G	0.000	0.000	0.000	0.000	0.111	0.000
H	0.000	0.029	0.000	0.000	0.000	0.000
Locus 204						
A	0.000	0.000	0.000	0.000	0.350	0.000
B	0.038	0.000	0.000	0.000	0.250	1.000
C	0.308	0.529	0.375	0.375	0.300	0.000
D	0.000	0.088	0.000	0.000	0.000	0.000
E	0.654	0.382	0.500	0.625	0.050	0.000
G	0.000	0.000	0.000	0.000	0.050	0.000
H	0.000	0.000	0.125	0.000	0.000	0.000
Locus 250						
A	0.077	0.206	0.000	0.286	0.500	0.667
B	0.115	0.147	0.375	0.286	0.150	0.000
C	0.000	0.029	0.000	0.000	0.000	0.000
D	0.692	0.324	0.500	0.429	0.150	0.000
E	0.000	0.000	0.000	0.000	0.000	0.333
F	0.077	0.294	0.125	0.000	0.150	0.000
G	0.000	0.000	0.000	0.000	0.050	0.000
H	0.038	0.000	0.000	0.000	0.000	0.000
Locus 377						
A	0.846	0.471	0.500	1.000	0.050	0.333
E	0.115	0.176	0.500	0.000	0.050	0.000
F	0.038	0.088	0.000	0.000	0.200	0.167
J	0.000	0.000	0.000	0.000	0.000	0.333
K	0.000	0.088	0.000	0.000	0.000	0.000
N	0.000	0.000	0.000	0.000	0.050	0.000
O	0.000	0.176	0.000	0.000	0.400	0.167
P	0.000	0.000	0.000	0.000	0.050	0.000
Q	0.000	0.000	0.000	0.000	0.200	0.000
Locus 2158						
A	0.000	0.059	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.167
C	0.042	0.059	0.000	0.000	0.000	0.000
D	0.458	0.324	0.000	0.500	0.000	0.667
E	0.000	0.000	0.000	0.000	0.444	0.000
F	0.000	0.088	0.250	0.000	0.111	0.000
G	0.083	0.265	0.375	0.375	0.000	0.000
H	0.083	0.000	0.000	0.000	0.000	0.167
I	0.333	0.147	0.375	0.000	0.000	0.000
J	0.000	0.059	0.000	0.000	0.000	0.000
L	0.000	0.000	0.000	0.125	0.444	0.000

Table 2. Expected heterozygosity (number of alleles in parentheses) and probability of identity in Italian wolf samples (AR – Tuscan Apennines; AB – Central-southern Apennines; VC – Alta Maremma; FR – Alpes Maritimes).

	Heterozygosity				Probability of Identity			
Locus	AR	AB	VC	FR	AR	AB	VC	FR
109	0.621 (5)	0.562 (4)	0.406 (3)	0.594 (3)	0.197	0.236	0.388	0.248
123	0.640 (4)	0.723 (6)	0.594 (3)	0.719 (4)	0.182	0.125	0.248	0.130
204	0.476 (3)	0.566 (3)	0.594 (3)	0.469 (2)	0.357	0.277	0.248	0.392
250	0.494 (5)	0.744 (5)	0.594 (3)	0.653 (3)	0.282	0.110	0.248	0.194
377	0.269 (3)	0.701 (5)	0.500 (2)	0.000 (1)	0.555	0.128	0.375	1.000
2 158	0.663 (5)	0.785 (7)	0.656 (3)	0.594 (3)	0.170	0.076	0.193	0.248
All Loci	0.527	0.680	0.557	0.505	3.4×10^{-4}	8.7×10^{-6}	4.3×10^{-4}	6.1×10^{-4}

Table 3. Genetic variation at 6 microsatellite loci and deviation from Hardy-Weinberg equilibrium. N, sample size; A, mean number of alleles per locus; H_o, observed heterozygosity (direct count); H_e, Hardy-Weinberg expected heterozygosity; HWE, significance of chi-square test for Hardy-Weinberg equilibrium without pooling; HWE_p, significance of chi-square test for Hardy-Weinberg equilibrium with pooling; SE, standard error; n. s., not significant.

Subpopulation/Population	N	A (SE)	H_o	H_e (SE)	HWE	HWE_p
AR – Tuscan Apennines	13	4.0 (0.4)	0.579	0.527 (0.061)	n. s.	n. s.
AB – Central-southern Apennines	17	5.0 (0.6)	0.676	0.680 (0.038)	n. s.	n. s.
VC – Alta Maremma	4	2.8 (0.2)	0.792	0.557 (0.036)	n. s.	n. s.
FR – Alpes Maritimes	4	2.7 (0.4)	0.652	0.505 (0.106)	n. s.	n. s.
Total (Italian population)	38	6.0 (0.7)	0.664	0.644 (0.040)	n. s.	n. s.
SP – Spain	10	4.8 (0.5)	0.683	0.693 (0.023)	n. s.	n. s.
SL – Slovakia	3	2.3 (0.4)	0.389	0.435 (0.097)	n. s.	n. s.

Table 4. Comparison of genetic variation at 5 microsatellite loci among different wolf populations and related canid species (N, sample size; A, mean number of alleles per locus ± standard error; H_e, Hardy-Weinberg expected heterozygosity ± standard error).
[a] recomputed from single locus frequencies data.

Species/Population	N	A	H_e	Reference
Canis lupus				
Italy	38	5.4 ± 0.4	0.619 ± 0.039	this study
Spain (Asturias)	10	5.2 ± 0.5	0.713 ± 0.013	this study
Canada (Northwest Territories)	24	6.0 ± 1.2	0.714 ± 0.063	Roy et al. (1994)[a]
Canada (Alberta)	20	5.2 ± 0.6	0.709 ± 0.027	Roy et al. (1994)[a]
USA (Montana)	66	5.0 ± 0.4	0.659 ± 0.055	Forbes and Boyd (1997)[a]
USA (Yellowstone National Park)	31	5.4 ± 0.7	0.686 ± 0.074	Forbes and Boyd (1997)[a]
Canis simensis	42	2.6 ± 0.7	0.167 ± 0.011	Gottelli et al. (1994)[a]
Canis lupus f. familiaris	40	6.8 ± 0.8	0.714 ± 0.075	Gottelli et al. (1994)[a]

loci for the overall Italian population equals –0.039), indicating breeding among non-relatives. Departures from the Hardy-Weinberg equilibrium for the different samples proved not significant at all loci. However, as shown in table 3, a recent colonized area (FR) and a strongly fluctuating population (VC) showed a marked excess of heterozygotes, although statistically not significant. Linkage disequilibrium for each pair of loci was confirmed by a probability test analysis in GENEPOP.

Referring to the restricted analysis (over 5 loci), mean H_e for the Italian sample was 0.619 ± 0.039, with a mean number of alleles per locus (A) of 5.4 ± 0.4. A comparison with other wolf populations and with two canid species (Ethiopian wolf, *Canis simensis*, and domestic dog, *Canis lupus* f. *familiaris*) is shown in table 4.

Levels of differentiation among subpopulations and populations were detected by comparing allelic and genotypic frequency distributions across loci. Significant levels of divergence within the Italian range were obtained only from the comparison between AR and AB samples (allelic data: Fisher exact test, $p = 0.00044$; genotypic data: G-test $p = 0.00042$). On the other hand, the whole Italian population showed both a genic and a genotypic statistically significant divergence from the Spanish and the Slovakian samples (allelic data: Fisher exact test, $p < 0.001$; genotypic data: G-test $p < 0.001$). Single-locus comparisons allow discrimination of subpopulations due to the presence of private alleles. Both AR and AB subpopulations showed exclusive alleles at different loci. All the alleles but one (allele H at locus 204) present in the VC sample belong to the AB subpopulation also. On the other hand, the FR sample has alleles present in both AR and AB populations, except for three alleles at locus 123, one exclusive to AR and two to AB, respectively, and for allele L at locus 2158, absent in other Italian samples. Mean D_{AS} (proportion of alleles not shared) within the Italian population was 0.466, whereas the mean values of derived D'_{AS} for subpopulation pairs ranged between 0.049 and 0.162 (Tab. 5).

Nei's unbiased distances were lower than 0.2 for all the comparisons among Italian samples (Tab. 6 a, below diagonal), and higher than 0.6 for all inter-population pairwise comparisons (Tab. 6 b, below diagonal). In the former case, the minimum values were obtained between AB and VC samples (0.051) and between AR and FR samples (0.054). On the basis of Nei's unbiased distance, a cophenetic tree may be plotted (Fig. 2).

F_{ST} values accounted for the proportion of total variation due to diversity between samples. Overall, F_{ST} for the whole Italian population was 0.053, a low value considering that a value of zero expresses the iden-

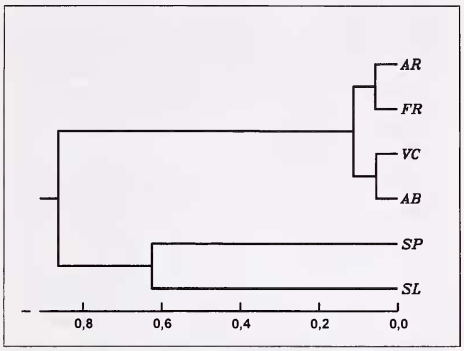

Fig. 2. Upgma phenogram of wolf populations based on Nei's unbiased genetic distances.

Table 5. Pairwise genetic distances by Shared Alleles (D'_{AS})

Subpopulation	AR	AB	VC
AR – Tuscan Apennines	*	*	*
AB – Central-southern Apennines	0.049	*	*
VC – Alta Maremma	0.070	0.061	*
FR – Alpes Maritimes	0.062	0.089	0.162

Table 6. Nei's unbiased genetic distance (below diagonal) and pairwise F_{ST}-values (above diagonal) among Italian subpopulations (a) and among European wolf populations (b).

a)

Subpopulation	AR	AB	VC	FR
AR – Tuscan Apennines		0.058	0.071	0.051
AB – Central-southern Apennines	0.094		0.022	0.042
VC – Alta Maremma	0.081	0.051		0.121
FR – Alpes Maritimes	0.054	0.093	0.162	

b)

Population	IT	SP	SL
IT – Italy		0.199	0.262
SP – Spain	0.764		0.181
SL – Slovakia		0.851	0.610

tity of allele frequencies among all subpopulations. F_{ST} values obtained for each subpopulation and population pair are shown in table 6 a and 6 b, respectively (above diagonal). The multilocus estimate of Nm for the overall Italian population gave a number of about 2.0 migrants per generation, which suggests a relevant amount of gene flow among subpopulations. When calculating Nm between the two most representative subpopulations, AR and AB, a value of 1.7 was obtained.

A positive correlation was found, using Mantel's test, only between D'_{AS} and geographical distance (Spearman rank correlation coefficient, $p = 0.038$), suggesting the presence of moderate isolation by distance. No significant correlation was found for Nei's distance and F_{ST}.

Discussion

Italian population samples showed a high intra-group diversity, as both H_e and P_{id} were relevant within each subpopulation. Although the standard error was sometimes considerable, due to the small sample size, the A was high for most loci even in small samples. Mean heterozygosity over 5 loci proved very close to the values obtained for North American populations (Roy et al. 1994; Forbes and Boyd 1997), and also the mean number of alleles per locus was completely comparable. This agrees with allozymic data, whose level of heterozygosity for the Italian population was found to be relatively high (Randi et al. 1993).

Comparing Italian wolf with a related species population, Ethiopian wolf (Gottelli et al. 1994), which went through prolonged isolation, a vast difference in heterozygosity calculated over the same loci is evident.

All samples fitted Hardy-Weinberg expectations, whether the χ^2-test was performed with or without pooling. Excess of heterozygotes in strongly fluctuating or recently colonized subpopulations, VC and FR, proved high but not significant. This may be due to random assembling of founder genotypes occupying new territories.

High Hardy-Weinberg expected heterozygosity, in comparison with the one observed in Italian samples, may arise from limited outbreeding, as confirmed by the negative F_{is} value. Breeding between unrelated individuals is a common trend in natural wolf populations (Smith et al. 1996), nevertheless high levels of induced mortality may enhance the natural turnover of pack members and favour outbreeding.

The most immediate indicator of genetic differentiation is allele frequency distribution. A significant level of divergence among Italian samples was found over all 6 loci only for the AB–AR pair, comparing both allelic and genotypic frequencies. As microsatellites are particularly sensitive to allele frequency differentiation, such differences may not be considered on their own a proof of genetic isolation.

Looking at allele frequency distribution, generally, the highest was the frequency across populations, and the widest was spatial diffusion. Several rare alleles were present, with occasional local specificity.

Examining the presence of single alleles, AR and AB samples showed a moderate level of diversity due to the presence of private alleles (5 for AR and 10 for AB). VC and FR samples appear compatible with a possible derivation from the other two subpopulations, but they also have exclusive alleles. The allele H at locus 204, present in the VC sample, was never found before in wolf individuals, whereas it proved the prevalent allele in dog samples (data not shown). A possible wolf dog hybridization event among the ancestors of the female individual presenting such an allele cannot be excluded. On the contrary, allele L at locus 2158 in the Alpine sample was found in other European wolves (e. g. in Spain) and might be present also at low frequency in the Italian population, but it was not detected in this work as a consequence of the limited sample size.

Both D_{AS} and Nei's unbiased distance values confirm the expected origin of the Alpine subpopulation from the northern Apennines. The allelic pattern of the VC sample, combined with measures of dis-

tance, is compatible with colonization from southern regions (AB). The area represents the most northern tail of the Tyrrhenian coast subpopulation, which is supposed to have maintained links up to the first half of the last century with the central-southern subpopulation and possibly have restored them in the last few decades. Data obtained in the present study seem to confirm this hypothesis. The overall F_{ST} value was small (0.053), suggesting very limited structuring of the Italian population.

Gene flow is relevant as, with more than 1 migrant per generation, differences among subpopulations are reduced, balancing the effect of genetic drift (Slatkin 1987). A value of 1.7, derived from the AR and AB subpopulations, is more reliable with respect to the estimate for the whole Italian population. This is because these subpopulations represent areas where the species was probably never eradicated, and they are close enough to maintain a sufficient level of gene flow. These aspects make the assumption of migration-drift equilibrium (Slatkin 1993) a plausible one.

Evidence for isolation by distance throughout the Italian population was found comparing D'_{AS} with geographic distances between different subpopulations. Historical factors and the geographic shape of the region played a key role in establishing a continuous and directional gene flow across the peninsula.

Summarizing, we have pointed out that the Italian wolf population constitutes a well-defined and viable natural population, where a high gene flow guarantees a sufficient genetic exchange among different areas. The origin of the nuclei settling in Alpes Maritimes should be attributed to movements of dispersing individuals from the northern Apennines, as suggested by the similarity between Alpine samples and specimens from the Tuscan Apennines. The Tyrrhenian subpopulation may have restored its continuity with the central Apennines, but human-caused mortality continuously threatens its stability, favouring high turnover and eventually outbreeding, at least in the peripheral zones of the wolf range.

Overall microsatellite diversity is substantial and comparable to that in North American populations. Inbreeding depression seems to be far from threatening Italian wolves. However demographic factors, difficult to predict, may affect population viability more than genetic aspects, producing dramatic changes especially in local situations. Therefore it would be advisable to maintain the genetic flow high across the peninsula, in order to balance the effect of local bottlenecks.

A long-term differentiation between Alpine and southern subpopulations may be expected as consequence of isolation by distance, while a progressive enlargement of the northern range along the Alps will progressively bring the Italian wolf closer to the Balkan populations.

The set of six polymorphic microsatellite loci used in this work represented an effective tool for investigating the actual genetic status of wild wolf populations. The low probability of identity between individuals (in the order of 10^{-4} to 10^{-6}) reveals a high resolution power in resolving pedigrees, i. e., for kinship analysis.

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